Sites in the Diyne–Ene Bicyclic Core of Neocarzinostatin Chromophore Responsible for Hydrogen Abstraction from DNA†

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ABSTRACT: The antitumor antibiotic neocarzinostatin exhibits its main drug action by abstracting hydrogen from DNA deoxyribose with consequent strand breakage or related lesions. All biological activities of the drug derive solely from a nonprotein chromophoric substance (NCS-chrom) consisting of a novel epoxy–bicyclo–diyne–ene system. Thiol or sodium borohydride activates NCS-chrom into a labile, reactive species that induces DNA damage but causes inactivation of the drug in the absence of the target DNA. Mass spectrometric studies indicate that the isolated thiol-activated NCS-chrom product in the presence of DNA has the same molecular weight as the thiol-inactivated NCS-chrom product in the absence of DNA. No deuterium is incorporated into the chromophore from the deuterium-labeled sulfhydryl group. Since three deuterium atoms can be incorporated into the drug by treatment with sodium borodeuteride without DNA, adding an unlabeled DNA under parallel conditions permitted the ready identification of the activated NCS-chrom product that abstracted hydrogen from the DNA. Not only does the activated NCS-chrom product have the same structure as the inactivated drug without DNA, but two of the incorporated deuterium atoms have been substituted by hydrogen. With the aid of NMR spectrometry, the two replaced hydrogen atoms are found to be incorporated into the C-2 and C-6 positions of the bicyclo–diyne–ene ring of NCS-chrom and are derived neither from borodeuteride nor from the hydroxyl functions of the solvents. In accord with current proposals, the two hydrogens incorporated into the drug may come from closely opposed sites on the complementary strands of the DNA at which the drug is bound. Our findings are consistent with a diradical mechanism in which carbon-centered radicals at C-2 and C-6 of the activated drug result in abstraction of hydrogen atoms either from the deoxyribose moiety of DNA or, in the absence of DNA, from some other source, but not from the hydrogen attached to the sulfur of thiols under the conditions described.

Neocarzinostatin (NCS)[1] is an antitumor antibiotic protein that expresses its biological activity by damaging DNA deoxyribose. All biological function of the drug results from a labile nonprotein chromophoric substance; the protein acts only as a carrier to protect the chromophore against degradation [see Goldberg (1987) for a review]. The chromophore of NCS (NCS chrom) can be isolated (Napier et al., 1979) and is made up of four components: a 2-hydroxy-5-methoxy-7-methyl-1-naphthoate, a 2,6-dideoxy-2-(methylamino)galactose, a five-membered cyclic carbonate ring, and a highly strained 12-carbon unit which features an epoxybicyclo[7.3.0]dodecadienediyne nucleus (NCS-chrom A in Figure 1) (Napier et al., 1981; Hensens et al., 1983; Edo et al., 1985). The fourth moiety is believed to be responsible for damaging DNA during the reaction with the drug (Napier & Goldberg, 1983; Koide et al., 1986). NCS-chrom is the first natural compound found to possess the novel bicyclo–diyne–ene ring. The related calichemicins and esperamicins, which have been reported to be among the most potent antitumor agents known, have recently also been shown to possess a central core containing a bicyclo–diyne–ene system (Golik et al., 1987a,b; Lee et al., 1987a,b).

The activation of NCS-chrom for DNA damage reaction requires a thiol or sodium borohydride (Kappen & Goldberg, 1978; Goldberg et al., 1981; Kappen et al., 1988). The thiol-activated[2] NCS-chrom can abstract a hydrogen atom from C-5′ of deoxyribose of thymidylate in DNA. The abstracted hydrogen is incorporated into the drug in a nonexchangeable form under both aerobic and anaerobic conditions (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985). Recently, hydrogen abstraction has also been found from the C-1′ position of deoxyribose of deoxycytidylate in DNA (Kappen et al., 1988). NCS-chrom can also be irreversibly inactivated by preincubation with thiol (Kappen & Goldberg, 1978). A number of chemical studies have been initiated to identify the reactive species of NCS-chrom generated upon interaction with thiols (Napier et al., 1981; Napier & Goldberg, 1983; Albers-Schonberg et al., 1980; Hensens et al., 1983; Sheridan & Gupta, 1981a,b). The chemical reactions of NCS-chrom with methylmercaptan, methyl thioglycolate, and sodium borohydride in the absence of DNA have been studied more extensively (Napier et al., 1981; Albers-Schonberg et al., 1980; Hensens et al., 1983). The characterization of the reaction products by NMR and mass spectrometric techniques

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[1] Abbreviations: NCS, neocarzinostatin; NCS-chrom, nonprotein chromophore of neocarzinostatin; FAB MS, fast atom bombardment mass spectrometry; MS/MS, high-performance tandem mass spectrometry; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance spectroscopy; HPLC, high-pressure liquid chromatography.

[2] The term "activated" drug is used in this paper to refer to NCS-chrom that has been activated by thiol or borohydride and reacts with DNA to produce DNA deoxyribose damage. "Inactivated" drug is used for NCS-chrom that was treated with thiol or borohydride in the absence of DNA.

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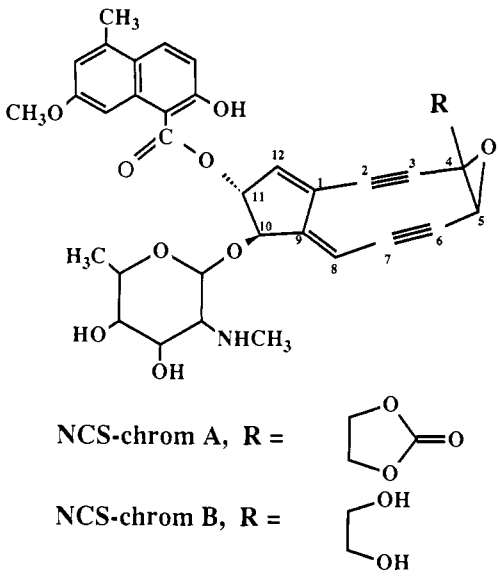


FIGURE 1: Structure of NCS-chrom. The absolute stereochemistry at C-10 and C-11 is depicted as *R,R* in accord with a model proposed by Schreiber (Schreiber & Kiessling, 1988).

has shed light on the mechanism of the irreversible inactivation of the drug (Hensens et al., 1983; Myers, 1987). However, the drug product after reaction with DNA has not been characterized. Nor has the inactivation reaction been carried out in the solvents and buffers used in the DNA reaction. Nevertheless, valuable models for the activation and inactivation of NCS have been proposed (Kappen & Goldberg, 1985; Favaudon, 1982; Myers, 1987). The activation mechanism recently proposed by Myers (1987) is the most detailed, but it is entirely based on the as yet unproved assumption that the product of activated NCS-chrom with DNA is equivalent to its inactivation product. Despite the similar chromatographic behaviors on HPLC of the activated and inactivated drug products (Kappen & Goldberg, 1985), their exact relationship was not known.

In this study we have characterized the activated product of NCS-chrom produced by thiol and sodium borohydride in the presence of DNA. By using a reverse isotope labeling technique, we have been able to identify the activated NCS-chrom product and to clarify further the mechanisms of drug activation and DNA damage. In particular, it has been possible to locate the exact active carbon sites of the drug that can abstract hydrogen from DNA, thus providing support for a mechanism involving a diradical form of the drug.

Experimental Procedures

Materials. NCS-chrom was extracted from the holoprotein and stored at −70 °C in methanol as described (Chin et al., 1987). Phage λ DNA with tritium (8.9 cpm/pmol) at the C-5′ position of thymidine was prepared as described (Chin et al., 1987). Sonicated calf thymus DNA was prepared as previously reported (Chin et al., 1984).

DNA–NCS-chrom Reaction. When glutathione was used to activate NCS-chrom, the reaction was performed anaerobically as described (Chin et al., 1987). When sodium borodeuteride was substituted for thiol, [5′-^{3}H]thymidine-labeled DNA (10%) was mixed with calf thymus DNA (90%) to give a final concentration of 0.63 mM, and NCS-chrom was added at a DNA phosphorus to drug ratio of 20. The mixture also contained sodium citrate at a final concentration of 6 mM and diethylenetriaminepentaacetic acid at a final concentration of 1 mM. In order to ensure that NCS-chrom was complexed to DNA, water was added so that the content of methanol (from NCS-chrom) did not exceed 15%. The solution was then lyophilized to dryness, and solid sodium borodeuteride, at a final concentration of 30 mM, and solid Tris-HCl, pH 7.0 [prepared as described (Chin et al., 1984)], at a final concentration of 62.5 mM, were combined with the dried ingredients in the dark. Deuterium oxide was then added as solvent to redissolve the mixture and to initiate the NCS-chrom–DNA reaction. The final pH was 8.0. The reaction solution was kept aerobically at room temperature in the dark for 2 hours before product analysis was performed.

In the inactivation of NCS-chrom by sodium borohydride/borodeuteride without DNA, the lyophilization step was omitted, and the reaction was carried out in 50% methanol with 50% water (when deuteriated solvent was needed, CH_3OD and deuterium oxide were used) in order to reduce the spontaneous degradation of NCS-chrom. Sodium borohydride/borodeuteride was freshly dissolved in water/deuterium oxide before the reaction and was added to the NCS-chrom solution followed by Tris-HCl buffer (prepared in water/deuterium oxide). The pH of the reaction solution rose as far as 9.5 ± 0.2 before finally equilibrating at 8.0. The amounts of the other ingredients remained the same as described above.

Product Analysis. The reacted solution was lyophilized and redissolved in a small amount of water. Methanol and absolute ethanol were then added to a final ratio of water:methanol:ethanol of 8:8:84. The solution was kept at 0 °C for 2 h to precipitate poly- and oligonucleotides. The supernatant fluid was lyophilized to dryness and redissolved in water before analysis by reverse-phase HPLC on a Rainin Microsorb C 18 (5 μm, 1 × 25 cm) column with a Beckman Model 332 gradient liquid chromatograph system. The column was eluted first for 5 min with aqueous 5 mM ammonium acetate (pH 5), followed by an 80-min convex gradient of 0–80% methanolic 5 mM ammonium acetate (pH 5) at a flow rate of 3 mL/min. A peak of radioactivity (^{3}H from C-5′), which was eluted at about 29 min for the reaction with sodium borodeuteride and at about 65 min for the reaction with glutathione, was lyophilized and further purified by HPLC isocratically on an Altex Ultrasphere ODS (5 μm, 0.46 × 25 cm) column at a flow rate of 1 mL/min. For the reaction with sodium borodeuteride, 30% methanolic 5 mM ammonium acetate (pH 5) was used, and the material was eluted between 15 and 17 min. For the reaction with glutathione, 55% methanolic 5 mM ammonium acetate (pH 5) was used, and the radioactive compound was eluted between 20 and 22 min. The materials were collected and evaporated for analyses by fast atom bombardment mass spectrometry (FAB MS) on a MAT 731 mass spectrometer and for high-performance tandem mass spectral (MS/MS) measurements on a JEOL HX110/HX110 spectrometer at the Mass Spectrometry Facility at the Massachusetts Institute of Technology (Sato et al., 1987). Low-resolution MS and collision-induced decomposition (CID) MS/MS measurements were performed at 1:1000 resolution in both MS-1 and MS-2. High-resolution MS measurements were made at 1:10 000 resolution. Glycerol or 3-nitrobenzyl alcohol was used as a matrix.

The same materials were also collected for NMR spectroscopy except that most samples had a final additional HPLC purification using 30% methanol in water acidified (pH values of 3.5–4.0) by a few drops of formic acid as eluant. The material elutes at about 10 min. Deuterium oxide was used as a solvent, and tetramethylsilane was used as an internal standard in the NMR studies. ^{1}H NMR spectra were obtained at 25 °C on a FT 500-MHz instrument at the Francis Bitter

National Magnet Laboratory at the Massachusetts Institute of Technology.

RESULTS

Activation by Glutathione

Previous findings by Hensens et al. (1983) indicate that the inactivation of NCS-chrom by thiol treatment under acidic conditions leaves the naphthalenecarboxylic acid, the aminogalactose, and the five-membered cyclic carbonate ring intact; it produces chemical changes only on the epoxybicyclo-[7.3.0]dodecadienediyne nucleus. NMR and mass spectrometric studies on the inactivated drug products (Hensens et al., 1983) suggest that (i) the epoxide ring is opened and one exchangeable proton is attached to the opened epoxide oxygen, (ii) there is considerable rearrangement of the unsaturated bicyclo ring system, (iii) one thiol anion is added to the C-12 position of the bicyclo ring, and (iv) reduction by two hydrogen atoms occurs on the unsaturated bicyclo–diyne–ene system. In summary, the inactivation of NCS-chrom by thiol leads to a final product in which one thiol molecule (a thiol anion with an exchangeable proton) and two nonexchangeable hydrogen atoms have been incorporated.

When DNA is a target in the drug reaction, DNA damage by NCS-chrom is enhanced at least 1000-fold by thiol. The activated NCS-chrom is able to abstract hydrogen anaerobically or aerobically from DNA into a nonexchangeable form (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985). As shown in Figure 2a, HPLC analysis of the supernatant solution after precipitation of DNA from a glutathione-activated NCS-chrom reaction containing a mixture of calf thymus DNA and [5′-^{3}H]thymidine-labeled λ DNA reveals coincident peaks of radioactivity and fluorescence, the latter due to the naphthalenecarboxylic acid group of the drug. Thiol-inactivated NCS-chrom is unable to abstract 5′-^{3}H from DNA but produces the same amount of UV-absorbing and fluorescing material with the same retention time (Kappen & Goldberg, 1985). These results imply that the inactivated species is structurally similar to the activated species that has abstracted the hydrogen from DNA.

Positive-ion fast atom bombardment mass spectrometric (FAB MS) studies of the isolated chromophore peak containing the abstracted 5′-^{3}H indicate that the material consists of one molecule of NCS-chrom, one molecule of glutathione, and two atoms of hydrogen and therefore suggest that both activated and inactivated drug products are compositionally the same. Two sets of peaks, a protonated molecular ion at m/z 969 with its sodium cation adduct at m/z 991 and a second protonated molecular ion at m/z 943 with its sodium cation adduct at m/z 965, were repeatedly obtained from separate preparations. The material at m/z 969 is consistent with a product of NCS-chrom A that has incorporated one glutathione molecule (M_r 307) and two hdyrogen atoms. The substance at m/z 943 is equivalent to the decarbonylation product of the former material (Figure 1). A decarbonylation form of NCS-chrom at the five-membered cyclic carbonate ring has been known as NCS-chrom B and is found to be present as a minor component (10%) in all preparation of NCS studied (Napier et al., 1981). It is, therefore, not surprising to observe a decarbonylation form coexisting with the parent five-membered cyclic carbonate material in a degraded activation product of the drug. Although high-resolution mass spectrometric analyses of the two sets of peaks could not be obtained due to relatively weak peak intensities, the coexistence of the decarbonylation form with the five-membered cyclic carbonate form actually supports the conclusion that both sets

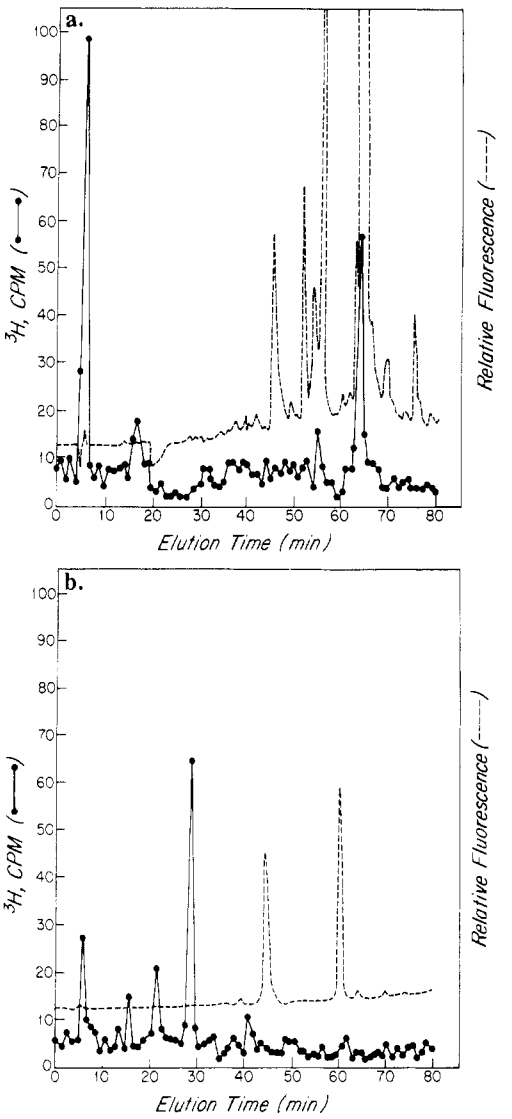


FIGURE 2: Abstraction of 5′-^{3}H from DNA into NCS-chrom which is activated by (a) glutathione or (b) sodium borodeuteride. The reaction conditions for (a) and (b) are described under Experimental Procedures. Total amount of the reaction solution used in (a) was 5 mL, and 0.33% (v/v) of the collected fractions was taken for radioactivity measurement. Total volume for reaction (b) was 10 mL, and 0.67% (v/v) of the eluted solution was counted for radioactivity. HPLC conditions are indicated under Experimental Procedures.

of peaks are derivatives of NCS-chrom[3] rather than merely impurities.

It is interesting to note that in the glutathione-activated DNA reaction there was no difference in enrichment at D_1 and D_2 whether water or deuterium oxide was used as solvents, indicating that deuterium atoms were not incorporated into the inactivated drug. This result suggests that neither of the two hydrogen atoms that have been abstracted and covalently bound to the inactivated drug comes from the exchangeable mercapto hydrogen, which is usually considered to be a very good hydrogen donor for carbon-centered radicals. This finding is in agreement with other reports (Albers-Schonberg et al., 1980; Hensens et al., 1983; Hensens and Goldberg, unpublished results). In fact, the first reported molecular formula of the NCS chrom (Albers-Schonberg et al., 1980) had to be revised (Hensens et al., 1983) downward by two

[3] Since the decarbonylation product is in great excess over the parent form, it is not known when and under what conditions the decarbonylation form is generated.

hydrogens due to the incorrect assumption that reduction by thiol had not occurred since the molecular weight was the same whether or not the solvent was deuteriated. NMR study of the inactivated drug produced by deuteriothioglycolate also shows that all resonances are still present (Hensens et al., 1983; Hensens and Goldberg, unpublished results).

Accordingly, in the absence of a labeled donor of the hydrogens involved in the reductive process, it was not possible to identify the exact positions where reduction occurs on the drug in the thiol reaction. Such information can be obtained by analogy with the reaction with sodium borohydride. On the basis of the NMR data of the inactivated drug products, the reaction with sodium borohydride is essentially comparable to that with thiol except that the addition of a thiol anion is replaced by a hydride on C-12 (Hensens et al., 1983). By using sodium borodeuteride, it was found that three deuterium atoms are incorporated at the C-12, C-2, and C-6 positions of the epoxybicyclo[7.3.0]dodecadienediyne nucleus (Hensens et al., 1983; Hensens and Goldberg, unpublished results) (details are presented in the next section). Therefore, it seems reasonable to assume that in the thiol-activated reaction the reduction occurs at C-2 and C-6, the same positions as with sodium borodeuteride.

Activation by Sodium Borohydride

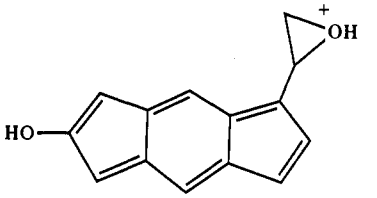
Although positive-ion FAB MS studies of the NCS-chrom–DNA reaction in glutathione clearly indicate that the activated NCS-chrom product that has abstracted hydrogen from DNA is compositionally the same as the inactivated drug product, direct evidence is needed to verify that the isolated material is not merely a product resulting from a competing inactivation process. NCS-chrom is a very labile substance and would not be expected to be fully utilized in the formation of DNA lesions. The higher the ratio of DNA to drug, the higher is the efficacy of DNA damage per molecule of drug. When the ratio of DNA phosphorus to drug was 16, about 64% of NCS-chrom was utilized in the production of measureable DNA lesions (Povirk & Goldberg, 1983). Some of the rest of the NCS-chrom was either inactivated by the thiol or degraded spontaneously under the basic conditions, and presumably, some was involved in producing DNA lesions that were subsequently repaired by the thiol. It is expected, therefore, that there will be more than one degradation product of the drug after reaction with DNA. To directly verify that the mass spectrometric results represent an activated NCS-chrom product that has abstracted hydrogen from DNA, deuterium isotope labeling became a necessity. However, not only is the synthesis of 5′,5′-D_2 DNA complex, but also 100% labeling with deuterium of all the nucleotides of DNA might be difficult, if not impossible, to achieve. Thus, an alternative reverse deuterium labeling method was designed. By using sodium borodeuteride as the activation reagent, deuterium oxide as a solvent, and unlabeled DNA in a NCS-chrom–DNA reaction, it is possible to determine if hydrogen from DNA is incorporated into the spent drug. A ratio of DNA to drug of 20 was used to minimize the competing inactivation reactions. Since the inactivated NCS-chrom should bear three nonexchangeable deuteriums and one proton under such a circumstance, the activated drug that has abstracted hydrogen from DNA should have one or more of the nonexchangeable deuterium atoms substituted by hydrogen. By employing this method, the activated drug product can be easily identified by MS or ^1H NMR among the inactivated species which may have the same structure but cannot be separated.

Figure 2b shows a HPLC chromatogram of a reaction similar to the one in Figure 2a except that sodium borodeuteride substitutes for glutathione as the drug-activating agent. The labeled peak containing abstracted tritium, which elutes at about 29 min, corresponds only with the UV absorbance. The absence of fluorescence indicates a loss of the naphthalenecarboxylic acid functionality. The much shortened retention time, compared with 65 min for the glutathione-activated NCS-chrom product, also suggests that the material is more hydrophilic, in agreement with the presumed loss of the hydrophobic naphthalene group.

Table I: Positive-Ion FAB MS and MS/MS Data for the Isolated Parent NCS-chrom and Its Products[a]

material	FAB MS of $(M + H)^+$ (m/z)	CID MS/MS of $(M + H)^+$ (m/z)			
		S	C	N	X[b]
NCS-chrom	660	160	86[c]	215	
inactivated NCS-chrom					
$NaBH_4$ in H_2O/CH_3OH	450	160	86		211
$NaBH_4$ in D_2O/CH_3OD	451	160	86		212
$NaBD_4$ in H_2O/CH_3OH	452	160	86		213
$NaBD_4$ in D_2O/CH_3OD	453	160	86		214
activated NCS-chrom					
DNA in $NaBD_4/D_2O$ (major peak)	451	160	86		212

[a] The activated and inactivated NCS-chrom products are from treatments with sodium borohydride/borodeuteride in hydrogen/deuteriated solvents. Abbreviations: S = protonated anhydro amino sugar ($C_7H_{14}O_3N$), C = dehydro cyclic carbonate ($C_3H_2O_3$), N = naphthoyl ($C_{13}H_{11}O_3$), and X = fragment X. [b] Fragment X is interpreted as the species which has lost the amino sugar, H_2O, and CO_2, proposed as shown below for the unlabeled compound; this structure presumes stereospecificity on the addition of H(D) and loss of H at C-12. An alternative structure for X having an α,β unsaturated aldehyde at position 4 and CH(D,H) at position 12 would have less rigid stereochemical requirements but would not be so well stabilized by conjugation as is X. [c] Edo et al. (1985) reported m/z 88 in their two-sector linked scan (SIMS) spectrum with NCS-chrom. Because of the improved accuracy in mass assignment provided by the four-sector instrument, the value given here is more likely correct.



m/z 211

Under similar experimental conditions, the inactivation of NCS-chrom by sodium borohydride/sodium borodeuteride without DNA exhibits an HPLC elution profile between 20 and 80 min that is similar to Figure 2b in terms of UV absorbance and fluorescence.[4] The material eluting at about 29 min, for which a corresponding fluorescence is lacking, was isolated and further investigated by positive-ion FAB MS, high-performance tandem mass spectrometry (MS/MS), and NMR spectrometry. Figure 3a shows the FAB CID MS/MS spectrum of the $(M + H)^+$ ion, m/z 660.2, of NCS-chrom A. Figure 3b shows the FAB CID MS/MS spectrum of the unlabeled inactivated product. Table I summarizes the observed mass spectrometric data for the original NCS-chrom, the inactivated drug products, and the activated drug product in the presence of DNA. The inactivation reactions of the drug were carried out in (a) sodium borohydride with an unlabeled solvent, (b) sodium borohydride with a solvent enriched with deuterium on its hydroxyl functionality, (c) sodium boro-

[4] The several UV-absorbing peaks between 10 and 20 min of elution in Figure 2b, which were missing in the absence of DNA, result from released bases (mainly thymine) and oligodeoxynucleotides.

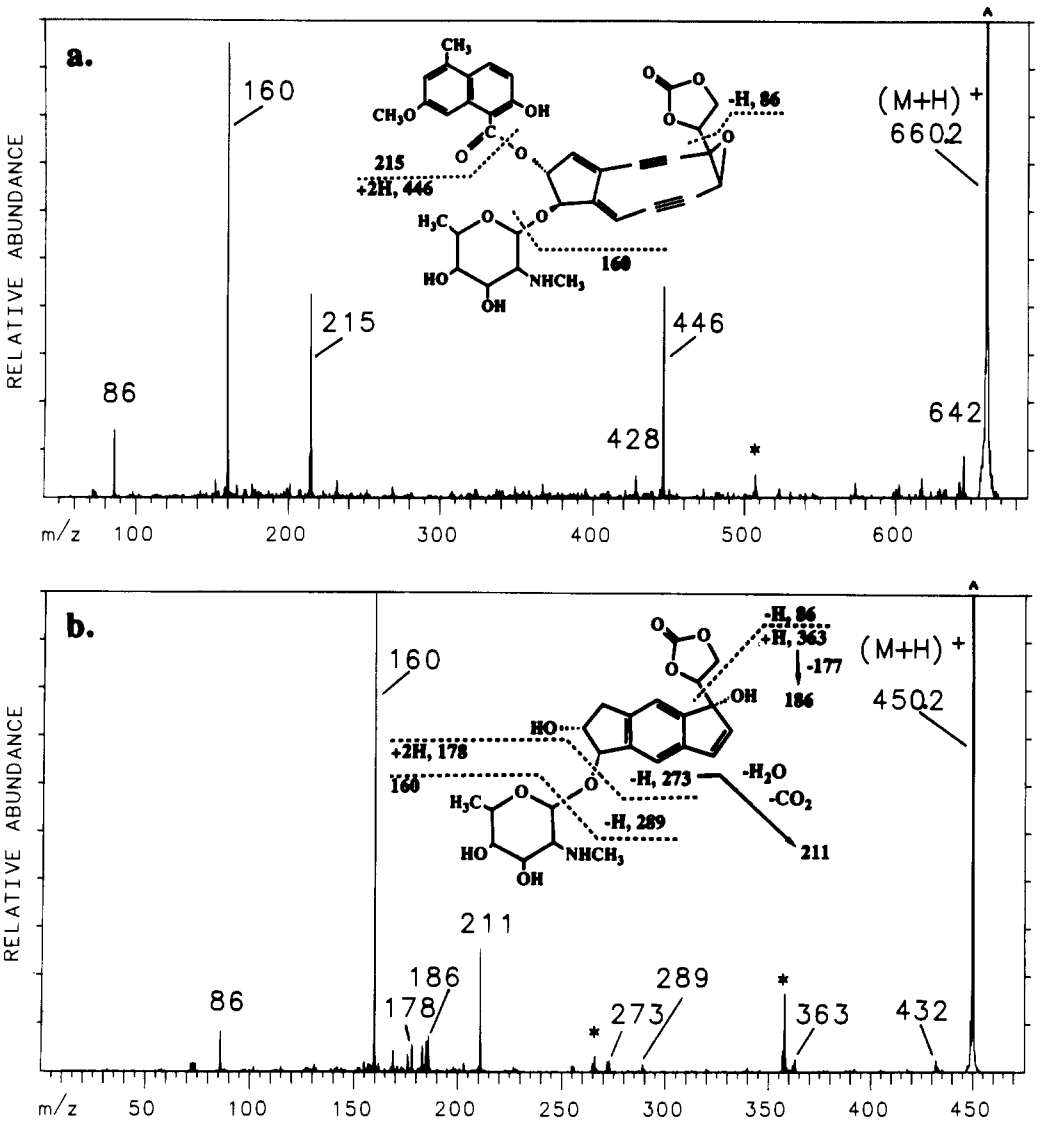


FIGURE 3: (a) FAB CID MS/MS spectrum of the $(M + H)^+$ ion, m/z 660.2, of NCS-chrom A. (b) FAB CID MS/MS spectrum of the $(M + H)^+$ ion, m/z 450.2, of the product obtained from NCS-chrom inactivated by reduction with $NaBH_4$ in H_2O/CH_3OH. Peaks marked with an asterisk arise from matrix-related cluster ions at the nominal mass selected by MS-1.

Table II: High-Resolution Exact Mass Measurements and the Corresponding Elemental Compositions of NCS-chrom and Its Products[a]

material	exact mass (m/z) deterd	exact mass (m/z) calcd	elemental composition
NCS-chrom	660.2054	660.2081	$C_{35}H_{34}O_{12}N$
inactivated NCS-chrom[b]	452.1892	452.1890	$C_{22}H_{26}D_2O_9N$
activated NCS-chrom[c]	451.1828	451.1827	$C_{22}H_{27}DO_9N$

[a] The inactivated and activated NCS-chrom products are from treatments with sodium borodeuteride in hydrogen/deuterium solvents. [b] Treatment with sodium borodeuteride with unlabeled solvent. [c] Treatment with sodium borodeuteride in the presence of DNA with D_2O.

Table III: Partial ^1H NMR Assignments of NCS-chrom Products from Treatments with Sodium Borohydride[a]

assignment	NCS-chrom product −naphthoate[b]	NCS-chrom product +naphthoate[c]
S5′-CH_3	1.05 (3 H, d, J = 6.4)	1.27 (3 H, d, J = 6.5)
S2′-$NHCH_3$	2.57 (3 H, s)	3.00 (3 H, s)
H-5	6.17 (1 H, d, J = 6.1)	6.35 (1 H, d, J = 5.5)
H-6	6.88 (1 H, d, J = 6.1)	6.97 (1 H, d, J = 5.5)
H-8	7.24 (1 H, s)	7.42 (1 H, s)
H-2	7.35 (1 H, s)	7.66 (1 H, s)

[a] Chemical shifts are given in ppm downfield of internal tetramethylsilane. Abbrevations: S = amino sugar, d = doublet, s = singlet, J = coupling constant in hertz. [b] Data are from the present study and were recorded in D_2O. [c] Data are from previous studies (Hensens et al., 1983; Hensens and Goldberg, unpublished results) and were recorded in CD_3CO_2D. Reaction was performed in methanolic acetic acid solution.

deuteride with an unlabeled solvent, and (d) sodium borodeuteride with a solvent enriched with deuterium on its hydroxyl functionality. The activation of the drug in the presence of DNA was performed under the same conditions as in (d). Table II shows the exact masses measured for the products determined by high-resolution mass spectrometry and their calculated elemental compositions. Figure 4a–c shows partial ^1H NMR spectra of the unlabeled and two and three deuterium-labeled inactivated chromophore products, and Figure 4d shows the activated chromophore product after reduction by $NaHD_4$ in D_2O in the presence of DNA. Table III lists chemical shifts of some identified peaks from ^1H NMR studies. Proton resonances are assigned by a close comparison with the previously reported NMR data of an inactivation reaction (Hensens et al., 1983; Hensens and Goldberg, unpublished results). Several important conclusions can be drawn from these data:

(*1*) *Structural Comparison of the Activated and the Inactivated Chromophore Products.* The high-resolution mass

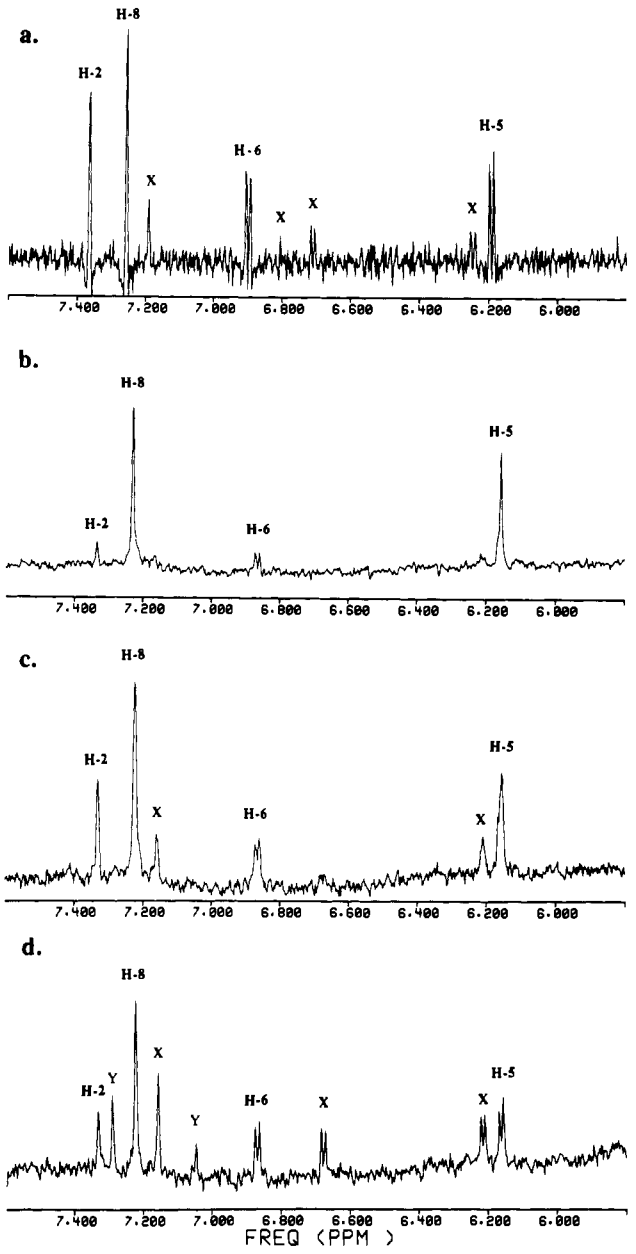


FIGURE 4: Partial ^1H NMR spectra of (a) the inactivated NCS-chrom obtained from treatment with $NaBH_4$ in H_2O/CH_3OH, (b) the inactivated NCS-chrom product from treatment with $NaBD_4$ in D_2O/CH_3OD, (c) the inactivated NCS-chrom product from treatment with $NaBD_4$ in H_2O/CH_3OH, and (d) the activated NCS-chrom product from treatment with $NaBD_4$ in D_2O in the presence of DNA. Peaks marked with X and Y are unknown.

spectrometric data (Table II) clearly show that the sodium borodeuteride activated NCS-chrom that has abstracted hydrogen from DNA is compositionally the same as the inactivated chromophore produced by sodium borodeuteride alone, except that there are different numbers of deuterium atoms incorporated into each species. Figure 3b shows the FAB CID MS/MS spectrum of the $(M + H)^+$ ion, m/z 450.2, obtained for the inactivated chromophore after reduction by $NaBH_4$ in H_2O/CH_3OH. MS/MS spectra of the products obtained by $NaBH_4$ reduction in D_2O/CH_3OD and by $NaBD_4$ reduction of either the inactivated or activated compound were the same, except that appropriate mass shifts were observed for the fragments containing the deuterium label [fragment X (Table I) and m/z 432, 363, 289, 273, and 186]. The MS/MS measurements of the activated and inactivated chromophore products therefore indicate that their structures are identical. The most direct proof comes from the ^1H NMR studies which show the same resonance patterns for each species. All of these data suggest that both activation and inactivation reactions lead to the same final degradation product of NCS-chrom.

(*2*) *Chemical Composition of the Product.* By comparing the mass spectrometric data of the parent NCS-chrom and its products in Table I, it is apparent that the most probable chemical composition shown in Table II for the NCS-chrom product consists of 1 NCS-chrom + 4 H − 1 naphthoate group. The loss of the naphthoate group, which also occurs as a minor reaction under acidic conditions, as previously reported (Napier & Goldberg, 1983), is indicated not only by the absence of the fragment at m/z 215 in MS/MS spectra but also by the absence of the proton resonances of naphthoate by ^1H NMR spectroscopy. Since essentially quantitative reduction of esters can be achieved by excess sodium borohydride (Gerrard, 1961), it seems likely that reduction of the naphthoate ester group of NCS-chrom by the 1000-fold excess of sodium borohydride accounts for its cleavage. Under the acidic reaction conditions employed by Napier and Goldberg (1983), sodium borohydride decomposes rapidly and may not have been able to complete the reduction of the ester group on NCS-chrom.

(*3*) *Localization of the Four Additional Hydrogen Atoms.* The MS and NMR data for the NCS-chrom product generated under the $NaBH_4$ conditions used in this paper are essentially in agreement with those previously reported for the inactivated chromophore product that still contained the naphthoate group after the inactivation reaction (Hensens et al., 1983; Hensens and Goldberg, unpublished results). It appears from the earlier results that one exchangeable proton is added at the opened epoxide ring to form an hydroxy functional group at the C-4 position and that three carbon-bound hydrogens are located at the C-2, C-6, and C-12 positions. The MS/MS studies of the NCS-chrom products prove that the activation and inactivation reactions leave the aminogalactose and the five-membered cyclic carbonate ring intact. All four hydrogen atoms, one exchangeable and three carbon bound, are added on the epoxy–bicyclo nucleus of the NCS-chrom as indicated by fragment X in Table I. NMR studies of the inactivated drug products show that most proton resonances of the epoxy–bicyclo nucleus are in agreement with the previous report (Figure 4, Table III), except that H-10, H-11, and H-12 are shifted, as expected, due to the loss of the naphthoate group. These results indicate that the chemical reaction that has occurred on the epoxy–bicyclo nucleus in the present study is the same as that involved in the formation of inactivated products that retain the naphthoate group. The ^1H NMR study of the inactivated NCS-chrom product formed by sodium borodeuteride in deuterium-enriched solvent (Figure 4b) shows that both resonances at H-2 and H-6 mostly disappear, and the resonance at H-5 is changed from a doublet into a singlet, as expected. This result, which is consistent with the previous one, clearly suggests that two of the three carbon-bound hydrogens are located at C-2 and C-6. Although the NMR spectra are too weak to identify the methylene protons on C-12 due to the low concentration of the material, it is reasonable to assign the third additional carbon-bound hydrogen to C-12 on the basis of the previous results.

(*4*) *Origins of the Three Carbon-Bound Hydrogen Atoms.* (*a*) *Inactivated NCS-chrom Product.* From Table I, it is clear that one of the three carbon-bound hydrogen atoms comes from the hydroxyl functional group of the solvent, and the remaining two carbon-bound hydrogen atoms come directly from borohydride. The ^1H NMR study of the NCS-chrom inactivated by sodium borodeuteride in an unlabeled solvent

(Figure 4c) shows that the H-5 resonance is a combination of a doublet and a singlet. Although precise integration could not be obtained due to the relatively weak signal to noise ratios, it still shows that both H-2 and H-6 contain more protons than the expected background (Figure 4b) and are about half as much as H-5, suggesting that the only carbon-bound hydrogen from solvent is not exclusively distributed on either C-2 or C-6.

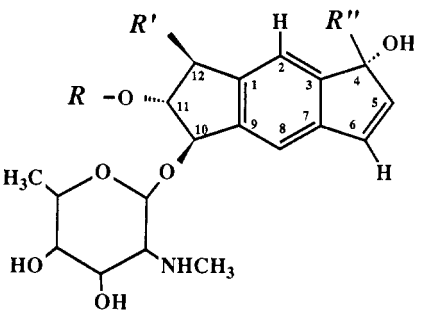
(*b*) *Activated NCS-chrom Product.* The most significant result shown in Table I is that two of the three incorporated deuterium atoms in the inactivated NCS-chrom product have been substituted by hydrogen atoms when DNA is present under otherwise similar conditions. Although the ^1H NMR spectrum of the activated species (Figure 4d) contains greater amounts of unknown peaks than the others (peaks marked with a Y are presently under investigation; peaks marked with an X appear at the same positions on some of the inactivated spectra), it still clearly shows that the H-5 resonance remains mostly as a doublet, indicating that H-6 is mainly hydrogen rather than deuterium. Integration data suggest that the enrichment of hydrogen for H-6 is about the same as for H-5. The integration of H-2 is less than H-6 but still indicates enrichment of hydrogen. Therefore, the two carbon-bound hydrogens resulting from reduction, which are DNA related, are presumably localized on C-2 and C-6, and the third incorporated carbon-bound hydrogen is added directly from borohydride onto C-12. Since the major function of the drug toward DNA is to cause sugar oxidation at C-5′ of thymidine, one of the DNA-related hydrogens that has been incorporated into the drug must come from the abstraction of 5′-H of thymidylate of DNA. It is clear that neither borohydride nor the hydroxyl functional group of the solvent donates the second hydrogen.

It should be noted that FAB MS analysis of the activated drug product in the presence of DNA shows some D_2 and D_3 enrichment in addition to the most abundant D_1 species whose $(M + H)^+$ appears at m/z 451. This is not surprising since more than one drug product in the DNA reaction is expected due to the competing inactivation reaction and the complex nature of the system. The intensity of the D_2 peak at m/z 452 of the activated product is about 50% of the major peak at m/z 451, and the relative abundance of the D_3 peak at m/z 453 is about 40%. Expected relative abundances based on natural heavy isotope levels would be 25% and 4%, respectively.

Discussion

Even before the recognition of the existence of NCS-chrom it was proposed that activation (in the presence of DNA) and inactivation of neocarzinostatin by thiols involved similar chemical processes (Kappen & Goldberg, 1978; Lewis et al., 1980). It was postulated that thiol activated the antibiotic in either situation to the same labile, possibly radical, form which in the absence of DNA could be quenched by the high thiol levels (Kappen & Goldberg, 1978). With the demonstration by NMR and mass spectroscopic studies that in acidic methanol methyl thioglycolate reacts with the chromophore at two distinct sites, one forming a thiol–chromophore adduct and the other undergoing the addition of two hydrogens (Hensens et al., 1983), it was proposed that, under conditions of chromophore-induced DNA degradation, the chromophore–thiol addition reaction at one site activates or exposes the second reactive site for reaction with DNA (Povirk & Goldberg, 1983). In the absence of DNA the second reactive site was presumably reduced by the methyl thioglycolate to the same final product.

In this paper we show that under conditions suitable for drug-induced DNA damage the two chromophore end products generated in the presence or absence of DNA are identical, whether the activating agent is a thiol or sodium borohydride. The activated form of NCS-chrom presumably is formed after the addition of one thiol anion or one hydride to the C-12 position of the chromophore (Hensens et al., 1983; Myers, 1987). This reactive intermediate undergoes a marked rearrangement (Hensens et al., 1983) presumably to a diradical at C-2 and C-6 (Myers, 1987) that can then either abstract hydrogen from DNA to form a final stable product or abstract hydrogen from elsewhere, borohydride or thiol reagent, in the absence of DNA to form the same product. As proposed by Myers (1987), ^1H and ^{13}C NMR data (Hensens et al., 1983; Hensens and Goldberg, unpublished results) support a tetrahydroindacene structure for the inactivated bicyclo–diyne–ene ring (Figure 5). The mechanism of aromatization of NCS-chrom, analogous to the enediyne → 1,4-benzenediyl rearrangement described by Bergman (Bergman, 1972; Lockhart et al., 1981; Lockhart & Bergman, 1981) and recently proposed in the activation of the structurally related antibiotics esperamicin and calichemicin to 1,4-dehydrobenzene diradicals (Golik et al., 1987a,b; Lee et al., 1987a,b), presumably involves the formation of a 2,6-dehydroindacene diradical. Accordingly, the abstraction of two DNA-related hydrogen atoms by activated NCS-chrom into C-2 and C-6 supports a DNA damage mechanism involing a diradical form of the drug.



1. Activated by glutathione.

R = 2-hydroxy-5-methoxy-7-methyl-1-naphthoate
R' = glutathione anion
R''= five-membered cyclic carbonate ring
or 1, 2-dihydroxy-ethyl

2. Activated by sodium borohydride.

R = H
R' = H
R''= five-membered cyclic carbonate ring

FIGURE 5: Proposed structures of the activated NCS-chrom products generated by treatment with glutathione or sodium borohydride in the presence of DNA.

In the case of calichemicin, a DNA damage mechanism involving a diradical form of the drug is consistent with the finding that this agent causes a high percentage of double-strand scissions in vitro (Zein et al., 1988). Neocarzinostatin, on the other hand, causes relatively few, if any, direct double-strand breaks; those that are formed can be ascribed to the relatively random placement of single-strand breaks within a few nucleotides of each other on complementary strands of the DNA. It has been found, however, that at least at certain sequences, such as AGC, abasic sites are generated on one strand of the DNA (at the C residue in AGC) and direct strand breaks are produced at the T residue opposite the A of AGC on the complementary strand (Kappen et al., 1988; Povirk & Houlgrave, 1988). In fact, double-strand breaks found after cleavage at the abasic site account for up to 25% of the total breaks formed (Povirk & Houlgrave, 1988). The formation of such bistranded lesions, presumably dependent

on DNA microstructure, can be rationalized on the basis of a diradical form of activated NCS-chrom in the minor groove of DNA. In keeping with such a proposal are the findings that drug abstracts hydrogen from C-5′ of thymidylate, resulting in a direct strand break with nucleoside 5′-aldehyde formation (Kappen et al., 1982; Kappen & Goldberg, 1983), and from C-1′ of deoxycytidylate, resulting in the formation of an abasic site (Kappen et al., 1988), consisting of 2-deoxyribonolactone with intact phosphodiester linkages (Kappen & Goldberg, 1988).

It is important to note that deuterium from solvent hydroxyl (and thus from the exchangeable sulfhydryl group) does not [contrary to the interpretation by Myers (1987)] become incorporated into the thiol-inactivated NCS-chrom under the conditions described. The specific source of the hydrogen that ends up at C-2 and C-6 of the chromophore is not known and is under investigation. In the case of calichemicin, deuterium incorporation into the drug was found only in CD_2Cl_2/CD_3OD but not in CH_2Cl_2/CD_3OD (Lee et al., 1987b). Analogously, it is possible that the acidic hydrogens on the carbon α to the sulfur in the thiol or an NH_2-substituted carbon in glutathione (Neta & Fessenden, 1971; Sjoberg et al., 1982) are the source of the hydrogen abstracted by the active form of NCS-chrom.

Our data indicate that in the borohydride-induced reaction solvent contributes a hydrogen to C-2/C-6 in addition to the hydrogen from the borohydride compound. C-2 and C-6 appear to both have the ability to accept the hydrogen either from the solvent or from the borohydride compound, suggesting that the diradical at C-2/C-6 is in equilibrium with a 2,6-dipolar resonance form. The transient difference in electronegativity at C-2 and C-6 could result from intramolecular electron transfer and result in facilitation of addition of polar reagents such as hydride and water.

Added in Proof

Myers et al. (1988) have recently verified by NMR the *R,R* stereochemistry at C-10 and C-11 shown in Figure 1.

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Photochemical Cross-Linking of Yeast tRNA^{Phe} Containing 8-Azidoadenosine at Positions 73 and 76 to the *Escherichia coli* Ribosome†

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ABSTRACT: The 3′-terminal -A-C-C-A sequence of yeast tRNA^{Phe} has been modified by replacing either adenosine-73 or adenosine-76 with the photoreactive analogue 8-azidoadenosine ($8N_3A$). The incorporation of $8N_3A$ into tRNA^{Phe} was accomplished by ligation of 8-azidoadenosine 3′,5′-bisphosphate to the 3′ end of tRNA molecules which were shortened by either one or four nucleotides. Replacement of the 3′-terminal A76 with $8N_3A$ completely blocked aminoacylation of the tRNA. In contrast, the replacement of A73 with $8N_3A$ has virtually no effect on the aminoacylation of tRNA^{Phe}. Neither substitution hindered binding of the modified tRNAs to *Escherichia coli* ribosomes in the presence of poly(U). Photoreactive tRNA derivatives bound noncovalently to the ribosomal P site were cross-linked to the 50S subunit upon irradiation at 300 nm. Nonaminoacylated tRNA^{Phe} containing $8N_3A$ at either position 73 or position 76 cross-linked exclusively to protein L27. When *N*-acetylphenylalanyl-tRNA^{Phe} containing $8N_3A$ at position 73 was bound to the P site and irradiated, 23S rRNA was the main ribosomal component labeled, while smaller amounts of the tRNA were cross-linked to proteins L27 and L2. Differences in the labeling pattern of nonaminoacylated and aminoacylated tRNA^{Phe} containing $8N_3A$ in position 73 suggest that the aminoacyl moiety may play an important role in the proper positioning of the 3′ end of tRNA in the ribosomal P site. More generally, the results demonstrate the utility of $8N_3A$-substituted tRNA probes for the specific labeling of ribosomal components at the peptidyltransferase center.

Photoaffinity labeling has proven to be a useful method for identifying components of the *Escherichia coli* ribosome that are located in or near the two main tRNA binding sites (Ofengand, 1980; Ofengand et al., 1986). In most cases, such studies have been performed with aminoacyl-tRNA molecules containing photolabile substituents either on the aminoacyl moiety or on one of the several naturally occurring bases containing reactive thiol or amino groups. As the reagents used for tRNA modification are typically 10–20 Å in length—and often quite bulky—there is always a possibility that the substituents may perturb the structure of the tRNA molecule, interfere with proper tRNA–ribosome binding, or cross-link to ribosomal components at some distance from the actual site of tRNA interaction (Cooperman, 1980). Although photochemical reaction of a specific tRNA base with a unique ribosomal site to form a "zero-length" cross-link would be highly desirable in these investigations, such cross-links have been completely characterized only for the attachment of the 5′-anticodon base of tRNA$_1^{Val}$ to the cytosine residue at position 1400 of *E. coli* 16S rRNA (Prince et al., 1982) and to the equivalent base in yeast 18S rRNA (Ehresmann et al., 1984). The generality of the above approach is limited by the absence of suitable photoreactive moieties in the vast majority of tRNA molecules. In an effort to overcome this difficulty, while preserving the advantages of zero-length cross-linking, we have investigated the utility of replacing normal nucleosides with photolabile nucleoside analogues to produce tRNA derivatives which can be cross-linked to ribosomes under conditions that are unlikely to adversely affect tRNA structure, ribosome integrity, or tRNA–ribosome association.

The 8-azidopurine analogues developed by Czarnecki et al. (1979) are probably the most common photolabile nucleosides in use today. While they have been applied to the exploration of nucleotide binding sites in a wide variety of proteins (Haley, 1983), they have rarely been employed in the study of nucleic acids. We believe that the 8-azidopurines have great potential for elucidating tRNA–ribosome contacts in cases where they can be specifically incorporated into the RNA chain. In this report, we described the introduction of 8-azidoadenosine ($8N_3A$)[1] into positions 73 and 76 at the 3′ end of yeast tRNA^{Phe}. When bound to the ribosomal P site in the presence of poly(U), the modified tRNA^{Phe} molecules form essentially zero-length cross-links to the ribosome upon irradiation of the complexes with light of 300 nm. The particular components

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[1] Abbreviations: $8N_3A$, 8-azidoadenosine; pNp, nucleoside 3′,5′-bisphosphate; [$8N_3A73$]tRNA^{Phe} and [$8N_3A76$]tRNA^{Phe}, tRNA^{Phe} derivatives containing $8N_3A$ at positions 73 and 76, respectively; tRNA^{Phe}(–A), tRNA^{Phe}(–CA), tRNA^{Phe}(–CCA), and tRNA^{Phe}(–ACCA), tRNA^{Phe} from which one, two, three, or four 3′-terminal nucleotides have been removed, respectively; [^{32}P]tRNA, tRNA containing ^{32}P adjacent to the $8N_3A$ residue; AcPhe, *N*-acetylphenylalanyl; PEI, poly(ethylenimine); EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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